Process for th Fermentativ Preparation of L-Amino Acids using Strains of th Enterobacteriaceae Family

Field of the Invention

This invention relates to a process for the fermentative preparation of L-amino acids, in particular L-threonine, using strains of the Enterobacteriaceae family in which the open reading frame (ORF) with the designation yjgF is attenuated.

Prior Art

L-Amino acids, in particular L-threonine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and very particularly in animal nutrition.

It is known to prepare L-amino acids by fermentation of strains of Enterobacteriaceae, in particular Escherichia coli (E. coli) and Serratia marcescens. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as e.g. stirring and supply of oxygen, or the composition of the nutrient media, such as e.g. the sugar concentration during the fermentation, or the working up to the product form, by e.g. ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites, such as e.g. the threonine analogue α-amino-β-hydroxyvaleric acid (AHV), or are auxotrophic for metabolites of regulatory importance and produce L-amino acid, such as e.g. L-threonine, are obtained in this manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of strains of the Enterobacteriaceae family which produce L-amino acids, by amplifying individual amino acid biosynthesis genes and investigating the effect on the production. Summarizing information on the cell and molecular biology of Escherichia coli and Salmonella are to be found in Neidhardt (ed): Escherichia coli and Salmonella, Cellular and Molecular Biology, 2nd edition, ASM Press, Washington, D.C., USA.

Object of the Invention

The object of the invention is to provide new measures for improved fermentative preparation of L-amino acids, in particular L-threonine.

Summary of the Invention

The invention provides a process for the fermentative preparation of L-amino acids, in particular L-threonine, using microorganisms of the Enterobacteriaceae family which, in particular, already produce L-amino acids and in which at least the nucleotide sequence which codes for the yjgF ORF or alleles thereof is or are attenuated.

Detailed Description of the Invention

Where L-amino acids or amino acids are mentioned in the following, this means one or more amino acids, including their salts, chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-Threonine is particularly preferred.

The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity or

concentration of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by using a weak promoter or a gene or allele or ORF which codes for a corresponding enzyme or protein with a low activity or inactivates the corresponding enzyme or protein or gene or ORF and optionally combining these measures.

Open reading frame (ORF) describes a section of a nucleotide sequence which codes or can code for a protein or polypeptide or ribonucleic acid to which no function can be assigned according to the prior art. After assignment of a function to the nucleotide sequence section in question, it is in general referred to as a gene. Alleles are in general understood as meaning variants of a given gene. These are distinguished by differences in the nucleotide sequence.

The protein coded by a nucleotide sequence, i.e. an ORF, a gene or an allele, or the ribonucleic acid coded is in general called the gene product.

By attenuation measures, the activity or concentration of the corresponding protein according to the invention or also of proteins from the prior art is in general reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of the activity or concentration of the protein in the starting microorganism. The starting microorganism is understood as meaning the microorganism on which the inventive measures are carried out.

The process is characterized in that the following steps are carried out:

a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-

amino acid and in which the yjgF ORF or nucleotide sequences which code for it is or are attenuated,

b) concentration of the corresponding L-amino acid in the medium or in the cells of the microorganisms of the Enterobacteriaceae family, and optionally

c) isolation of the desired L-amino acid, constituents of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100 %) thereof optionally remaining in the product.

The microorganisms, in particular recombinant microorganisms, which the present invention provides can produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, optionally starch, optionally cellulose or from glycerol and ethanol. They are representatives of the Enterobacteriaceae family chosen from the genera Escherichia, Erwinia, Providencia and Serratia. The genera Escherichia and Serratia are preferred. Of the genus Escherichia in particular the species Escherichia coli and of the genus Serratia in particular the species Serratia marcescens are to be mentioned.

Suitable strains, which produce L-threonine in particular, of the genus Escherichia, in particular of the species Escherichia coli, are, for example

- Escherichia coli H4581 (EP 0 301 572)
- Escherichia coli KY10935 (Bioscience Biotechnology and Biochemistry 61(11):1877-1882 (1997)
- Escherichia coli VNIIgenetika MG442 (US-A-4278,765)
- Escherichia coli VNIIgenetika M1 (US-A-4.321.325)
- Escherichia coli VNIIgenetika 472T23 (US-A-5,631,157)

- Escherichia coli BKIIM B-3996 (US-A-5.175.107)
- Escherichia coli kat 13 (WO 98/04715)
- Escherichia coli KCCM-10132 (WO 00/09660)

Suitable L-threonine-producing strains of the genus Serratia, in particular of the species Serratia marcescens, are, for example

- Serratia marcescens HNr21 (Applied and Environmental Microbiology 38(6): 1045-1051 (1979))
- Serratia marcescens TLr156 (Gene 57(2-3): 151-158 (1987))
- Serratia marcescens T-2000 (Applied Biochemistry and Biotechnology 37(3): 255-265 (1992))

Strains from the Enterobacteriaceae family which produce L-threonine preferably have, inter alia, one or more genetic or phenotypic features chosen from the group consisting of: resistance to α-amino-β-hydroxyvaleric acid, resistance to thialysine, resistance to ethionine, resistance to α-methylserine, resistance to diaminosuccinic acid, resistance to α-aminobutyric acid, resistance to borrelidin, resistance to cyclopentane-carboxylic acid, resistance to rifampicin, resistance to valine analogues, such as, for example, valine hydroxamate, resistance to purine analogues, such as, for example, 6-dimethylaminopurine, a need for L-methionine, optionally a partial and compensatable need for L-isoleucine, a need for meso-diaminopimelic acid, auxotrophy in respect of threonine-containing dipeptides, resistance to L-threonine, resistance to threonine raffinate, resistance to L-homoserine, resistance to L-lysine, resistance to L-methionine, resistance to L-glutamic acid, resistance to L-aspartate, resistance to L-leucine, resistance to L-phenylalanine, resistance to L-serine, resistance to L-

cysteine, resistance to L-valine, sensitivity to fluoropyruvate, defective threonine dehydrogenase, optionally an ability for sucrose utilization, enhancement of the threonine operon, enhancement of homoserine dehydrogenase I-aspartate kinase I, preferably of the feed back resistant form, enhancement of homoserine kinase, enhancement of threonine synthase, enhancement of aspartate kinase, optionally of the feed back resistant form, enhancement of aspartate semialdehyde dehydrogenase, enhancement of phosphoenol pyruvate carboxylase, optionally of the feed back resistant form, enhancement of phosphoenol pyruvate synthase, enhancement of transhydrogenase, enhancement of the RhtB gene product, enhancement of the RhtC gene product, enhancement of the YfiK gene product, enhancement of a pyruvate carboxylase, and attenuation of acetic acid formation.

It has been found that microorganisms of the Enterobacteriaceae family produce L-amino acids, in particular L-threonine, in an improved manner after attenuation, in particular elimination, of the yjgF gene or open reading frame (ORF) or alleles thereof.

The nucleotide sequences of the genes or open reading frames (ORF) of Escherichia coli belong to the prior art and can also be found in the genome sequence of Escherichia coli published by Blattner et al. (Science 277: 1453-1462 (1997)).

The yjgF ORF of Escherichia coli K12 is described, inter alia, by the following data:

Description: open reading frame

Function: unknown function

Description: the open reading frame yjgF codes for a 15.1 kDa protein, the isoelectric point is 5.8; chromosomally located, for example in

E. coli K12 MG1655 it lies in the intergene region of the genes mgtA, which codes for an Mg2+ transport ATPase of P-Typ1, and the pyrI gene, which codes for the regulatory subunit of aspartate carbamoyltransferase

Reference: Wasinger VC. and Humphery-Smith I.; FEMS Microbiology Letters 169(2): 375-382 (1998)
Volz K.; Protein Science 8(11): 2428-2437 (1999)
Parsons et al.; Biochemistry 42(1): 80-89 (2003)

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The yjgF ORF from Salmonella typhimurium is described, inter alia, in the following reference: Enos-Berlage et al.; Journal of Bacteriology 180(24): 6519-6528 (1998)

The nucleic acid sequences can be found in the databanks of the National Center for Biotechnology Information (NCBI) of the National Library of Medicine (Bethesda, MD, USA), the nucleotide sequence databank of the European Molecular Biologies Laboratories (EMBL, Heidelberg, Germany or Cambridge, UK) or the DNA databank of Japan (DDBJ, Mishima, Japan).

For better clarity, the known sequence for the yjgF ORF of Escherichia coli is shown under SEQ ID No.1 and the sequence, which is also known, for the yjgF ORF of Salmonella typhimurium is shown under SEQ ID No. 11. The proteins coded by these reading frames are shown as SEQ ID No. 2 and SEQ ID No. 12.

The open reading frames described in the text references mentioned can be used according to the invention. Alleles of these open reading frames or genes, in particular from Enterobacteriaceae, which result from the degeneracy of the genetic code or due to mutations such as are described

below can furthermore be used. The use of endogenous genes or open reading frames is preferred.

"Endogenous genes" or "endogenous nucleotide sequences" are understood as meaning the genes or open reading frames alleles or nucleotide sequences present in the population of a species.

Suitable alleles of the yjgF ORF include those which contain neutral-function mutations or "sense mutations". These include, inter alia, those which lead to at least one (1) conservative amino acid exchange in the protein coded by them. The maximum number of conservative amino acid exchanges can relate to 2, 3, 5, 10, 20 but in no case more than 30 amino acids. By the conservative amino acid exchanges mentioned, the functional capacity is lowered or increased by 0% to not more than 24%, 20%, 10%, 5%, 3%, 2% or 1%.

In the case of aromatic amino acids, conservative exchanges are referred to when phenylalanine, tryptophan and tyrosine are exchanged for one another. In the case of hydrophobic amino acids, conservative exchanges are referred to when leucine, isoleucine and valine are exchanged for one another. In the case of polar amino acids, conservative exchanges are referred to when glutamine and asparagine are exchanged for one another. In the case of basic amino acids, conservative exchanges are referred to when arginine, lysine and histidine are exchanged for one another. In the case of acidic amino acids, conservative exchanges are referred to when aspartic acid and glutamic acid are exchanged for one another. In the case of amino acids containing hydroxyl groups, conservative exchanges are referred to when serine and threonine are exchanged for one another. All other amino acid exchanges are called non-conservative amino acid exchanges.

In the same way, those nucleotide sequences which code for variants of the proteins mentioned which additionally contain a lengthening or shortening by at least one (1) amino acid on the N or C terminus can also be used. This lengthening or shortening is not more than 30, 20, 10, 5, 3 or 2 amino acids or amino acid radicals.

Suitable alleles also include those which code for proteins in which at least one (1) amino acid is inserted (insertion) or removed (deletion). The maximum number of such changes, called indels, can relate to 2, 3, 5, 10, 20 but in no case more than 30 amino acids.

Suitable alleles furthermore include those which are obtainable by hybridization, in particular under stringent conditions, using SEQ ID No. 1 or SEQ ID No. 11 or parts thereof, in particular the coding regions or the sequences complementary thereto.

Instructions for identifying DNA sequences by means of hybridization can be found by the expert, inter alia, in the handbook "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology 41: 255-260 (1991)). The hybridization takes place under stringent conditions, that is to say only hybrids in which the probe and target sequence, i.e. the polynucleotides treated with the probe, are at least 70% identical are formed. It is known that the stringency of the hybridization, including the washing steps, is influenced or determined by varying the buffer composition, the temperature and the salt concentration. The hybridization reaction is in general carried out under a relatively low stringency compared with the washing steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996).

A buffer corresponding to 5x SSC buffer at a temperature of approx. 50°C - 68°C, for example, can be employed for the hybridization reaction. Probes can also hybridize here with polynucleotides which are less than 70% identical to the sequence of the probe. Such hybrids are less stable and are removed by washing under stringent conditions. This can be achieved, for example, by lowering the salt concentration to 2x SSC and optionally subsequently 0.5x SSC (The DIG System User's Guide for Filter Hybridisation, Boehringer Mannheim, Mannheim, Germany, 1995) a temperature of approx. 50°C - 68°C being established. It is optionally possible to lower the salt concentration to a concentration corresponding to 0.2x SSC or 0.1x SSC. Polynucleotide fragments which are, for example, at least 70% or at least 80% or at least 90% to 95% or at least 96% to 99% identical to the sequence of the probe employed can be isolated by increasing the hybridization temperature stepwise from 50°C to 68°C in steps of approx. 1 - 2°C. Further instructions on hybridization are obtainable on the market in the form of so-called kits (e.g. DIG Easy Hyb from Roche Diagnostics GmbH, Mannheim, Germany, Catalogue No. 1603558).

To achieve an attenuation, for example, expression of the genes or open reading frames or the catalytic properties of the enzyme proteins can be reduced or eliminated. The two measures can optionally be combined.

The reduction in gene expression can take place by suitable culturing, by genetic modification (mutation) of the signal structures of gene expression or also by the antisense-RNA technique. Signal structures of gene expression are, for example, repressor genes, activator genes, operators, promoters, attenuators, ribosome binding sites, the start codon and terminators. The expert can find information in this respect, inter alia, for example, in Jensen and Hammer (Biotechnology and Bioengineering 58: 191-195 (1998)), in Carrier und Keasling (Biotechnology Progress 15: 58-64

(1999)), Franch and Gerdes (Current Opinion in Microbiology 3: 159-164 (2000)) and in known textbooks of genetics and molecular biology, such as, for example, the textbook by Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) or that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990).

Mutations which lead to a change or reduction in the catalytic properties of enzyme proteins are known from the prior art. Examples which may be mentioned are the works of Qiu and Goodman (Journal of Biological Chemistry 272: 8611-8617 (1997)), Yano et al. (Proceedings of the National Academy of Sciences of the United States of America 95: 5511-5515 (1998)), Wente and Schachmann (Journal of Biological Chemistry 266: 20833-20839 (1991)). Summarizing descriptions can be found in known textbooks of genetics and molecular biology, such as e.g. that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

Possible mutations are transitions, transversions, insertions and deletions of at least one (1) base pair or nucleotide. Depending on the effect of the amino acid exchange caused by the mutation on the enzyme activity, "missense mutations" or "nonsense mutations" are referred to. Missense mutation leads to an exchange of a given amino acid in a protein for another, this being, in particular, a non-conservative amino acid exchange. The functional capacity or activity of the protein is impaired by this means and reduced to a value of 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5%. Nonsense mutation leads to a stop codon in the coding region of the gene and therefore to a premature interruption in the translation. Insertions or deletions of at least one base pair in a gene lead to "frame shift mutations", which lead to incorrect amino acids being incorporated or translation being interrupted

prematurely. If a stop codon is formed in the coding region as a consequence of the mutation, this also leads to a premature termination of the translation. Deletions of at least one (1) or more codons typically also lead to a complete loss of the enzyme activity.

Instructions on generation of such mutations are prior art and can be found in known textbooks of genetics and molecular biology, such as e.g. the textbook by Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

Suitable mutations in the gene, such as the deletion mutations mentioned by way of example (see SEQ ID No. 10), can be incorporated into suitable strains by gene or allele replacement.

A common method is the method, described by Hamilton et al. (Journal of Bacteriology 171: 4617-4622 (1989)), of gene replacement with the aid of a conditionally replicating pSC101 derivative pMAK705. Other methods described in the prior art, such as, for example, that of Martinez-Morales et al. (Journal of Bacteriology 181: 7143-7148 (1999)) or that of Boyd et al. (Journal of Bacteriology 182: 842-847 (2000)), can likewise be used.

It is also possible to transfer mutations in the particular genes or mutations which affect expression of the particular genes or open reading frames into various strains by conjugation or transduction.

More detailed explanations of the terms in genetics and molecular biology are found in known textbooks of genetics and molecular biology, such as, for example, the textbook by Birge (Bacterial and Bacteriophage Genetics, 4th ed.,

Springer Verlag, New York (USA), 2000) or the textbook by Berg, Tymoczko and Stryer (Biochemistry, 5th ed., Freeman and Company, New York (USA), 2002) or the handbook by Sambrook et al. (Molecular Cloning, A Laboratory Manual, (3 volume set), Cold Spring Harbor Laboratory Press, Cold Spring Harbor (USA), 2001).

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, with strains of the Enterobacteriaceae family to enhance one or more enzymes of the known threonine biosynthesis pathway or enzymes of anaplerotic metabolism or enzymes for the production of reduced nicotinamide adenine dinucleotide phosphate or enzymes of glycolysis or PTS enzymes or enzymes of sulfur metabolism, in addition to the attenuation of the yjgF ORF.

The term "enhancement" in this connection describes the increase in the intracellular activity or concentration of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes or open reading frames, using a potent promoter or a gene or open reading frame which codes for a corresponding enzyme or protein with a high activity, and optionally combining these measures.

By enhancement measures, in particular over-expression, the activity or concentration of the corresponding protein is in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on that of the wild-type protein or the activity or concentration of the protein in the starting microorganism.

For the production of L-amino acids, in particularly L-threonine, it may be advantageous, in addition to the attenuation of the yjgF ORF, at the same time for one or more of the genes chosen from the group consisting of

- the thrABC operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase (US-A-4,278,765),
- the pyc gene of Corynebacterium glutamicum which codes for pyruvate carboxylase (WO 99/18228),
- the pps gene which codes for phosphoenol pyruvate synthase (Molecular and General Genetics 231(2): 332-336 (1992)),
- the ppc gene which codes for phosphoenol pyruvate carboxylase (Gene 31: 279-283 (1984)),
- the pntA and pntB genes which code for transhydrogenase (European Journal of Biochemistry 158: 647-653 (1986)),
- the rhtB gene which imparts homoserine resistance (EP-A-0 994 190),
- the mqo gene which codes for malate:quinone oxidoreductase (WO 02/06459),
- the rhtC gene which imparts threonine resistance (EP-A-1 013 765),
- the thrE gene of Corynebacterium glutamicum which codes for the threonine export protein (WO 01/92545),
- the gdhA gene which codes for glutamate dehydrogenase (Nucleic Acids Research 11: 5257-5266 (1983); Gene 23: 199-209 (1983)),
- the hns gene which codes for the DNA-binding protein HLP-II (WO 03/004671),
- the pgm gene which codes for phosphoglucomutase (WO 03/004598),

the fba gene which codes for fructose biphosphate aldolase (WO 03/004664),

- the ptsH gene of the ptsHIcrr operon which codes for the phosphohistidine protein hexose phosphotransferase of the phosphotransferase system PTS (WO 03/004674),
- the ptsI gene of the ptsHIcrr operon which codes for enzyme I of the phosphotransferase system PTS (WO 03/004674),
- the crr gene of the ptsHIcrr operon which codes for the glucose-specific IIA component of the phosphotransferase system PTS (WO 03/004674),
- the ptsG gene which codes for the glucose-specific IIBC component (WO 03/004670),
- the lrp gene which codes for the regulator of the leucine regulon (WO 03/004665),
- the csrA gene which codes for the global regulator Csr (Journal of Bacteriology 175: 4744-4755 (1993)),
- the fadR gene which codes for the regulator of the fad regulon (Nucleic Acids Research 16: 7995-8009 (1988)),
- the iclR gene which codes for the regulator of central intermediate metabolism (Journal of Bacteriology 172: 2642-2649 (1990)),
- the mopB gene which codes for the 10 kd chaperone (WO 03/004669) and is also known by the name groES,
- the ahpC gene of the ahpCF operon which codes for the small sub-unit of alkyl hydroperoxide reductase (WO 03/004663),
- the ahpF gene of the ahpCF operon which codes for the large sub-unit of alkyl hydroperoxide reductase (WO 03/004663),

- the cysK gene which codes for cysteine synthase A (WO 03/006666),
- the cysB gene which codes for the regulator of the cys regulon (WO 03/006666),
- the cysJ gene of the cysJIH operon which codes for the flavoprotein of NADPH sulfite reductase (WO 03/006666),
- the cysI gene of the cysJIH operon which codes for the haemoprotein of NADPH sulfite reductase (WO 03/006666),
- the cysH gene of the cysJIH operon which codes for adenylyl sulfate reductase (WO 03/006666),
- the phoB gene of the phoBR operon which codes for the positive regulator PhoB of the pho regulon (WO 03/008606),
- the phoR gene of the phoBR operon which codes for the sensor protein of the pho regulon (WO 03/008606),
- the phoE gene which codes for protein E of the outer cell membrane (WO 03/008606),
- the pykF gene which codes for fructose-stimulated pyruvate kinase I (WO 03/008609),
- the pfkB gene which codes for 6-phosphofructokinase II (WO 03/008610),
- the malE gene which codes for the periplasmic binding protein of maltose transport (WO 03/008605),
- the sodA gene which codes for superoxide dismutase (WO 03/008613),
- the rseA gene of the rseABC operon which codes for a membrane protein with anti-sigmaE activity (WO 03/008612),

- the rseC gene of the rseABC operon which codes for a global regulator of the sigmaE factor (WO 03/008612),
- the sucA gene of the sucABCD operon which codes for the decarboxylase sub-unit of 2-ketoglutarate dehydrogenase (WO 03/008614),
- the sucB gene of the sucABCD operon which codes for the dihydrolipoyltranssuccinase E2 sub-unit of 2-ketoglutarate dehydrogenase (WO 03/008614),
- the sucC gene of the sucABCD operon which codes for the β-sub-unit of succinyl-CoA synthetase (WO 03/008615),
- the sucD gene of the sucABCD operon which codes for the α-sub-unit of succinyl-CoA synthetase (WO 03/008615),
- the adk gene which codes for adenylate kinase (Nucleic Acids Research 13(19): 7139-7151 (1985)),
- the hdeA gene which codes for a periplasmic protein with a chaperonin-like function (Journal of Bacteriology 175(23): 7747-7748 (1993)),
- the hdeB gene which codes for a periplasmic protein with a chaperonin-like function (Journal of Bacteriology 175(23): 7747-7748 (1993)),
- the icd gene which codes for isocitrate dehydrogenase (Journal of Biological Chemistry 262(22): 10422-10425 (1987)),
- the mglB gene which codes for the periplasmic, galactose-binding transport protein HLP-II (Molecular and General Genetics 229(3): 453-459 (1991)),
- the lpd gene which codes for dihydrolipoamide dehydrogenase (European Journal of Biochemistry 135(3): 519-527 (1983)),

- the aceE gene which codes for the E1 component of the pyruvate dehydrogenase complex (European Journal of Biochemistry 133(1): 155-162 (1983)),
- the aceF gene which codes for the E2 component of the pyruvate dehydrogenase complex (European Journal of Biochemistry 133(3): 481-489 (1983)),
- the pepB gene which codes for aminopeptidase B (Journal of Fermentation and Bioengineering 82: 392-397 (1996)),
- the aldH gene which codes for aldehyde dehydrogenase (E.C. 1.2.1.3) (Gene 99(1): 15-23 (1991)),
- the bfr gene which codes for the iron storage homoprotein (bacterioferritin) (Journal of Bacteriology 171(7): 3940-3947 (1989)),
- the udp gene which codes for uridine phosphorylase (Nucleic Acids Research 17(16): 6741 (1989)) and
- the rseB gene which codes the regulator of sigmaE factor activity (Molecular Microbiology 24(2): 355-371 (1997)),

to be enhanced, in particular over-expressed.

The use of endogenous genes or open reading frames is in general preferred.

It may furthermore be advantageous for the production of L-amino acids, in particular threonine, in addition to the attenuation of the yjgF ORF, for one or more of the genes chosen from the group consisting of

- the tdh gene which codes for threonine dehydrogenase (Journal of Bacteriology 169: 4716-4721 (1987)),
- the mdh gene which codes for malate dehydrogenase (E.C. 1.1.1.37) (Archives in Microbiology 149: 36-42 (1987)),

- the gene product of the open reading frame (orf) yjfA (Accession Number AAC77180 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA), WO 02/29080),
- the gene product of the open reading frame (orf) ytfP (Accession Number AAC77179 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA), WO 02/29080),
- the pckA gene which codes for the enzyme phosphoenol pyruvate carboxykinase (WO 02/29080),
- the poxB gene which codes for pyruvate oxidase (WO 02/36797)
- the aceA gene which codes for the enzyme isocitrate lyase (WO 02/081722),
- the dgsA gene which codes for the DgsA regulator of the phosphotransferase system (WO 02/081721) and is also known under the name of the mlc gene,
- the fruR gene which codes for the fructose repressor (WO 02/081698) and is also known under the name of the cra gene,
- the rpoS gene which codes for the sigma^{38} factor (WO 01/05939) and is also known under the name of the katF gene,
- the aspA gene which codes for aspartate ammonium lyase (aspartase) (WO 03/008607) and
- the aceB gene which codes for malate synthase A (WO 03/008603)

to be attenuated, in particular eliminated or for the expression thereof to be reduced.

In addition to attenuation of the yjgF ORF it may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms produced according to the invention can be cultured in the batch process (batch culture), the fed batch process (feed process) or the repeated fed batch process (repetitive feed process). A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/ Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and optionally cellulose, oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep

liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e.g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the abovementioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

The fermentation is in general carried out at a pH of 5.5 to 9.0, in particular 6.0 to 8.0. Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 25°C to 45°C, and preferably 30°C to 40°C. Culturing is continued until a maximum of L-amino acids or L-threonine has formed. This target is usually reached within 10 hours to 160 hours.

The analysis of L-amino acids can be carried out by anion exchange chromatography with subsequent ninhydrin derivation, as described by Spackman et al. (Analytical Chemistry, 30: 1190-1206 (1958)) or it can be carried out by reversed phase HPLC, as described by Lindroth et al. (Analytical Chemistry 51: 1167-1174 (1979)).

The process according to the invention is used for the fermentative preparation of L-amino acids, such as, for example, L-threonine, L-isoleucine, L-valine, L-methionine, L-homoserine and L-lysine, in particular L-threonine.

A pure culture of the Escherichia coli K-12 strain DH5α/pMAK705 was deposited as DSM 13720 on 8th September 2000 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty.

The present invention is explained in more detail in the following with the aid of embodiment examples.

The minimal (M9) and complete media (LB) for Escherichia coli used are described by J.H. Miller (A Short Course in Bacterial Genetics (1992), Cold Spring Harbor Laboratory Press). The isolation of plasmid DNA from Escherichia coli and all techniques of restriction, ligation, Klenow and alkaline phosphatase treatment are carried out by the method of Sambrook et al. (Molecular Cloning - A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press). Unless described otherwise, the transformation of Escherichia coli is carried out by the method of Chung et al. (Proceedings of the National Academy of Sciences of the United States of America 86: 2172-2175 (1989)).

The incubation temperature for the preparation of strains and transformants is 37°C. Temperatures of 30°C and 44°C are used in the gene replacement method of Hamilton et al.

Example 1

Construction of the deletion mutation of the yjgF ORF

Parts of the gene regions lying upstream and downstream of the yjgF ORF and parts of the 5' and 3' region of the yjgF ORF are amplified from Escherichia coli K12 using the polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the nucleotide sequence of the yjgF ORF and sequences lying upstream and downstream in E. coli K12 MG1655 (SEQ ID No. 1, Accession Number AE000495), the following PCR primers are synthesized (MWG Biotech, Ebersberg, Germany):

yjgF-1: 5' - TCGCGATCTGGTACTGTAAG - 3' (SEQ ID No. 3)

yjgF-2: 5' - CTGTACGTAAGGACCGATAG - 3' (SEQ ID No. 4)

yjgF-3: 5' - CGCTGTTCGTCGCTAATCTT - 3' (SEQ ID No. 5)

yjgF-4: 5' - GATCTTCTTCGGACCGATCA - 3' (SEQ ID No. 6)

The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated according to the manufacturer's instructions with "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A DNA fragment approx. 622 bp in size from the 5' region of the yjgF ORF, called yjgF5' and shown in SEQ ID No. 7 and a DNA fragment approx. 612 bp in size from the 3' region of the yjgF ORF, called yjgF3' and shown in SEQ ID No. 8, can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with Taq-DNA polymerase (Gibco-BRL, Eggenstein, Germany).

The PCR products are each ligated with the vector pCR2.1TOPO (TOPO TA Cloning Kit, Invitrogen, Groningen, The Netherlands) in accordance with the manufacturers instructions and transformed into the E. coli strain TOP10F'. Selection of plasmid-carrying cells takes place on

LB agar, to which 50 µg/ml ampicillin are added. After isolation of the plasmid DNA, the vector pCR2.1yjgF5' is cleaved with the restriction enzymes Ecl136II and XbaI and, after separation in 0.8% agarose gel, the yjgF5' fragment is isolated with the aid of the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). After isolation of the plasmid DNA the vector pCR2.1yjgF3' is cleaved with the enzymes EcoRV and XbaI and ligated with the yjgF5' fragment isolated. The E. coli strain DH5α is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar, to which 50 µg/ml ampicillin are added. After isolation of the plasmid DNA those plasmids in which the mutagenic DNA sequence shown in SEQ ID No. 9 is cloned are detected by control cleavage with the enzymes HindIII/ XbaI and StuI/ HincII. One of the plasmids is called pCR2.1TOPOΔyjgF.

Example 2

Construction of the replacement vector pMAK705ΔyjgF

The deleted yjgF ORF described in example 1 is isolated from the vector pCR2.1TOPOΔyjgF after restriction with the enzymes HindIII and XbaI and separation in 0.8% agarose gel, and ligated with the plasmid pMAK705 (Hamilton et al. (1989) Journal of Bacteriology 171, 4617 - 4622), which had been digested with the enzymes HindIII and XbaI. The ligation batch is transformed in DH5α and plasmid-carrying cells are selected on LB agar, to which 20 µg/ml chloramphenicol are added. Successful cloning is demonstrated after isolation of the plasmid DNA and cleavage with the enzymes HindIII/XbaI and PstI. The replacement vector formed, pMAK705ΔyjgF (= pMAK705deltayjgF), is shown in figure 1.

Example 3

Position-specific mutagenesis of the yjgF ORF in the E. coli strain MG442

The L-threonine-producing E. coli strain MG442 is described in the patent specification US-A-4.278.765 and deposited as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

For replacement of the chromosomal yjgF ORF with the plasmid-coded deletion construct, MG442 is transformed with the plasmid pMAK705ΔyjgF. The gene replacement is carried out by the selection method described by Hamilton et al. (1989) Journal of Bacteriology 171, 4617 – 4622) and is verified by standard PCR methods (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with the following oligonucleotide primers:

yjgF-1: 5' – TCGCGATCTGGTACTGTAAG – 3' (SEQ ID No. 3)

yjgF-4: 5' – GATCTTCTTCGGACCGATCA – 3' (SEQ ID No. 6)

After replacement has taken place, MG442 contains the form of the ΔyjgF allele shown in SEQ ID No. 10. The strain obtained is called MG442ΔyjgF.

Example 4

Preparation of L-threonine with the strain MG442ΔyjgF

MG442ΔyjgF is multiplied on minimal medium with the following composition: 3.5 g/l $Na_2HPO_4*2H_2O$, 1.5 g/l KH_2PO_4, 1 g/l NH_4Cl, 0.1 g/l $MgSO_4*7H_2O$, 2 g/l glucose, 20 g/l agar. The formation of L-threonine is checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l $(NH_4)_2SO_4$, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4*7H_2O$, 15 g/l $CaCO_3$, 20 g/l glucose are inoculated and the batch is incubated for 16 hours at 37ºC and 180 rpm on

an ESR incubator from Kühner AG (Birsfelden, Switzerland). 250 µl of this preculture are transinoculated into 10 ml of production medium (25 g/l $(NH_4)_2SO_4$, 2 g/l KH_2PO_4, 1 g/l $MgSO_4*7H_2O$, 0.03 g/l $FeSO_4*7H_2O$, 0.018 g/l $MnSO_4*1H_2O$, 30 g/l $CaCO_3$, 20 g/l glucose) and the batch is incubated for 48 hours at 37°C. After the incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer from Dr. Lange (Düsseldorf, Germany) at a measurement wavelength of 660 nm.

The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.

The result of the experiment is shown in Table 1.

Table 1

Strain	OD (660 nm)	L-Threonine
MG442	6.0	1.5
MG442ΔyjgF	6.3	2.1

Brief Description of the Figure:

- Figure 1: pMAK705ΔyjgF (= pMAK705deltayjgF)

The length data are to be understood as approx. data. The abbreviations and designations used have the following meaning:

- cat: chloramphenicol resistance gene
- rep-ts: temperature-sensitive replication region of the plasmid pSC101
- yjgF5´: part of the 5' region of the yjgF ORF and the region lying upstream
- yjgF3´: part of the 3' region of the yjgF ORF and the region lying downstream

The abbreviations for the restriction enzymes have the following meaning

- EcoRV: restriction endonuclease from Escherichia coli RY13
- Ecl136II: restriction endonuclease from Escherichia coli
- HincII: restriction endonuclease from Haemophilus influenzae R_C
- HindIII: restriction endonuclease from Haemophilus influenzae R_D
- PstI: restriction endonuclease from Providencia stuartii
- StuI: restriction endonuclease from Streptomyces tubercidicus
- XbaI: Restriction endonuclease from Xanthomonas badrii